Kinetic study of the photochemical changes of (ZZ)-bilirubin IX α bound to human serum albumin

Demonstration of (EZ)-bilirubin IX α as an intermediate in photochemical changes from (ZZ)-bilirubin IX α to (EZ)-cyclobilirubin IX α

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The present study was performed to elucidate why the photochemical reaction of (ZZ)-bilirubin bound to human serum albumin is singularly selective, and only one of the two (EZ)- and (ZE)-bilirubins, the (ZE)-isomer, is produced. In a kinetic study of the photochemical reaction *in vitro*, the sum of the relative rate constants of photochemical transformation of (EZ)-bilirubin into both (EZ)-cyclobilirubin and (ZZ)-bilirubin, with a significant preference for the former, was proved to be considerably larger than that of the transformation of (ZZ)-bilirubin into (EZ)-bilirubin. Therefore only one of the geometrical isomers, namely (ZE)-bilirubin, is apparently formed. It was concluded that (EZ)-bilirubin photochemically undergoes (EZ)-cyclization, i.e. structural photoisomerization, while bound to its high-affinity site on human serum albumin, and is an intermediate in the transformation of (ZZ)-bilirubin into (EZ)-cyclobilirubin.

The two quantitatively important photoproducts in bilirubin metabolism during phototherapy are (ZE)-bilirubin and (EZ)-cyclobilirubin, with a predominance of the former in Gunn rats (Onishi *et al.*, 1984b) and of the latter in human newborn infants (Onishi *et al.*, 1980b; Isobe, 1983). The interrelations between bilirubin and its geometric or structural photoisomers during photochemical reaction are not in parallel but rather in series, as follows: first of all, geometric photoisomerization, i.e. (EZ)- and (ZE)-bilirubin formation from (ZZ)-bilirubin followed by (EE)-bilirubin formation from either (EZ)- or (ZE)-bilirubin, takes place; subsequently, structural photoisomerization in which (EZ)-cyclobilirubin is formed from (EZ)-bilirubin occurs; lastly, geometric photoisomerization from (EZ)-cyclobilirubin to (EE)-cyclobilirubin takes place again (Onishi *et al.*, 1981, 1984a; Isobe *et al.*, 1983).

However, a different view has been presented as described below (McDonagh *et al.*, 1982a,b; McDonagh, 1983). The principal pathways for bilirubin photoproduct formation *in vivo* consist of three independent pathways: one going to the geometric isomerization (*E*-configuration), one going

to the structural isomerization (*E*-cyclization) and a third going to oxidation products. That is, (EZ)-bilirubin is not an intermediate in (EZ)-cyclobilirubin formation, and the direct formation from bilirubin is a concerted single-photon process proceeding via singlet bilirubin. Although, in experiments *in vitro*, photochemical configurational isomerization of bilirubin bound to serum albumin from guinea pig, rat, mouse and cow generates (EZ)- and (ZE)-bilirubin with a slight preference for the latter, with human serum albumin the reaction is singularly selective, and only one of the two possible (EZ)- and (ZE)-bilirubins is produced, namely the (ZE)-isomer. Thus complexing of bilirubin with serum albumin has a marked species-dependent influence on bilirubin photoisomerization.

We obtained almost the same results (Itoh *et al.*, 1983). In plasma of newborn infants during phototherapy, similar marked stereoselectivity and regioselectivity is clearly observed. That is, the concentration of the (EZ)- and (ZE)-bilirubins in serum of newborn infants during phototherapy may become as high as 20% of the total bilirubin present (Onishi *et al.*, 1980b; Isobe, 1983), whereas that of the (EZ)-cyclobilirubin is under 1% (Onishi *et al.*, 1982). On the other hand, in the biliary excre-

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Scheme 1. Geometric and structural isomerization of (ZZ)-bilirubin IX α (modified from Onishi et al., 1981, 1984a; Isobe et al., 1983)

→, Photochemical and thermal conversion; ←, photochemical conversion only; *, theoretical pathway, but not confirmed. The steric configurations of the hydrogen atom and the methyl group at C-2 and the methyl group at C-7 are not shown.

tion pattern of the photoproducts in newborn infants during phototherapy, the order of the quantitatively important bile pigments is as follows: (EZ)-cyclobilirubin is the most important, the (EZ)- and (ZE)-bilirubins are second and mono- and di-glucuronides are third. In the overall mechanism of phototherapy in the human newborn infant, the formation of structural isomers, that is (EZ)-cyclobilirubin occurs, considerably more slowly but is quantitatively more important than that of (EZ)- and (ZE)-bilirubin, as described previously (Onishi *et al.*, 1980b; Isobe, 1983).

A kinetic study on the photochemical interconversion between bilirubin and its photoisomers bound to human serum albumin in solution, followed by h.p.l.c., was used for the present investigation to demonstrate that (EZ)-bilirubin is indubitably an intermediate in the formation of (EZ)-cyclobilirubin from (ZZ)-bilirubin (Scheme 1).

Materials and methods

Photochemical experiments

Except where noted otherwise, in all experiments the solutions were deoxygenated with pure (99.99%) N₂. Several 200 μ l portions of the solution in Pyrex tubes were exposed to light. At different periods of light exposure, the tubes were successively transferred to the dark. Each portion was analysed by h.p.l.c. Light intensity, determined with a Minolta Air-Shields Fluoro-lite-meter 451 (Minolta Air-Shields), at the surface of the solution was 27.3 μ W $\cdot$ cm⁻² $\cdot$ nm⁻¹.

Except for photochemical experiments, all manipulations were carried out in the dark or photographic safe light and at room temperature.

Preparation for photochemical experiments

Bilirubin/human serum albumin. Solutions of bilirubin/human serum albumin were prepared by adding 50 μ l of a bilirubin solution, made up by dissolving 10mg of crystalline bilirubin in 10ml of 0.05M-NaOH, to 1.0ml of human serum albumin solution (2.0g/100ml) in 0.1M-sodium phosphate buffer, pH 7.4, after which 100 μ l of the same buffer was added. The solution was used to study the photochemical conversions of (ZZ)-bilirubin into (ZE)-bilirubin and/or (ZZ)-bilirubin into (EZ)-bilirubin and (EZ)-cyclobilirubin (Scheme 2).

(EZ)- and (ZE)-Bilirubin-enriched human serum albumin solution. Preparations highly enriched in (EZ)- and (ZE)-bilirubins were obtained by solvent extraction of crude photolysis products as described below. A trace of EDTA was added to saturated bilirubin solution in distilled chloroform. Photolysis under anaerobic conditions was carried out in stoppered 10ml Pyrex tubes located 3cm

above an array of four fluorescent tubes. Four 2.0ml portions of the solution were exposed to light in the tubes for 10min. The residues obtained after evaporation of the solvent *in vacuo* at room temperature by use of a centrifugal evaporator (Yamato) were mixed with methanol. The purpose of the following procedure is to avoid the contamination of (ZZ)-bilirubin as much as possible. The supernatant obtained from centrifugation for 5 min at 1000 rev./min was filtered rapidly on a membrane filter. The residues obtained from the supernatant by a similar evaporation procedure were dissolved in 400 μ l of 0.1M-phosphate buffer, pH 7.4, 150 μ l of the solution was added to 1ml of serum albumin solution (2.0g/100ml) and then the solution was kept in the dark at 4°C for 24h. The supernatant obtained from centrifugation for 5 min at 1000 rev./min was used for studies of the photochemical conversion of (EZ)-bilirubin into (ZZ)-bilirubin and (EZ)-cyclobilirubin.

Sample preparation for h.p.l.c.

Acetonitrile (1 vol.) and dimethyl sulfoxide (1 vol.) were added to 1 vol. of the sample, and the whole was vortex-mixed for 30s and then centrifuged for 5 min at approx. 1000g. A portion of the supernatant was injected into the chromatograph. The preparation was carried out immediately (within 6min) before injection. Equipment, preparation of calibration curve, peak assignments and pigments and reagents were as described previously (Onishi *et al.*, 1979, 1980a; Isobe & Onishi, 1981; Itoh *et al.*, 1983).

H.p.l.c.

Equipment. A Shimadzu LC-3AFG liquid chromatograph with an SPD-1 detector and Chromatopac C-R1A was used for all h.p.l.c. procedures. A Zorbax ODS column (25cm $\times$ 4.6mm) was used.

Chromatograph operation. The variable-wavelength detector was set at 455nm. The mobile phase was a mixture of acetonitrile, 0.01M-sodium phosphate buffer, pH 6.8, and dimethylformamide. Separation was achieved by using a gradient of acetonitrile (5–20%, v/v) (Itoh *et al.*, 1983). Assignments of (EZ)-/(ZE)-bilirubins were in accordance with the method of McDonagh *et al.* (1982a,b).

Scheme 2. Main photochemical reaction of (ZZ)-bilirubin IX α and its rate constants

Results and discussion

Kinetic study of (ZZ)-bilirubin-serum albumin complexes in solution

Transformation of (ZZ)-bilirubin into (ZE)-bilirubin (reaction i)

Kinetics for reaction (i) are as follows.

$$\frac{dB}{dt} = k_2 D - k_1 B$$

$$\frac{dD}{dt} = k_1 B - k_2 D$$

where B and D are percentages of B and D respectively.

For Laplace conversion:

$$(s + k_1)Q_1 - k_2 Q_2 = B_0$$

$$k_1 Q_1 - (s + k_2)Q_2 = 0$$

$$\begin{aligned} Q_1 &= \frac{\begin{vmatrix} B_0 & -k_2 \\ 0 & -(s + k_2) \end{vmatrix}}{\begin{vmatrix} s + k_1 & -k_2 \\ k_1 & -(s + k_2) \end{vmatrix}} \\ &= \frac{-B_0(s + k_2)}{-(s + k_1)(s + k_2) + k_1 k_2} = \frac{B_0 s + k_2 B_0}{s^2 + (k_1 + k_2)s} \end{aligned} \quad (1)$$

$$\begin{aligned} Q_2 &= \frac{\begin{vmatrix} s + k_1 & -B_0 \\ k_2 & 0 \end{vmatrix}}{\begin{vmatrix} s + k_1 & -k_2 \\ k_1 & -(s + k_2) \end{vmatrix}} \\ &= \frac{-k_2 B_0}{-(s + k_1)(s + k_2) + k_1 k_2} = \frac{k_2 B_0}{s^2 + (k_1 + k_2)s} \end{aligned} \quad (2)$$

where s is the parameter of Laplace conversion.

If $B = A_1 e^{-\alpha t} + A_2 e^{-\beta t}$ and Laplace conversion takes place:

$$Q_1 = \frac{A_1}{s + \alpha} + \frac{A_2}{s + \beta} = \frac{(A_1 + A_2)s + A_1\beta + A_2\alpha}{s^2 + (\alpha + \beta)s + \alpha\beta} \quad (3)$$

$\therefore$ from eqns. (1) and (3):

$$\alpha + \beta = k_1 + k_2 \quad \alpha\beta = 0$$

From $A_1 + A_2 = B_0$ and $A_1\beta + A_2\alpha = k_2 B_0$:

$$\beta = 0$$

$$\alpha = k_1 + k_2$$

$$A_2 = \frac{k_2}{k_1 + k_2} B_0$$

$$A_1 = \frac{k_1}{k_1 + k_2} B_0$$

Therefore:

$$B = \frac{k_1}{k_1 + k_2} B_0 e^{-(k_1 + k_2)t} + \frac{k_2}{k_1 + k_2} B_0$$

Similarly:

$$D = \frac{k_1 B_0}{k_1 + k_2} (1 - e^{-(k_1 + k_2)t}) \quad (4)$$

That is, when $t \rightarrow \infty$, B converges with $k_2 B_0 / (k_1 + k_2)$ and D converges with $k_1 B_0 / (k_1 + k_2)$.

If $B_0 = 100$:

$$\frac{k_2}{k_1 + k_2} = 0.76 \quad \frac{k_1}{k_1 + k_2} = 0.24$$

From eqn. (4):

$$\left(1 - \frac{k_1 + k_2}{k_1 B_0} D\right) = e^{-(k_1 + k_2)t}$$

$$\ln\left(1 - \frac{k_1 + k_2}{k_1 B_0} D\right) = -(k_1 + k_2)t$$

Duration of irradiation (s)	$1 - \frac{D}{24}$
0	1
6	0.46
12	0.17
24	0
36	0

A change of the $1 - D/24$ plotted against time on a semi-logarithmic scale is shown in Fig. 2.

$$\begin{aligned} k_1 + k_2 &= \frac{\ln 2}{5} \\ &= 1.39 \times 10^{-1} \\ k_1 &= 3.34 \times 10^{-2} \\ k_2 &= 1.06 \times 10^{-1} \end{aligned}$$

In the control experiment with a non-irradiated solution, a large peak of (ZZ)-bilirubin was separated. When photoirradiation was applied for a

Fig. 1. (a) Changes in percentages of (EZ)-bilirubin and (ZE)-bilirubin as a result of photochemical reaction after irradiation of (ZZ)-bilirubin bound to human serum albumin, and (b) changes in percentages of (ZZ)-bilirubin, (EZ)-cyclobilirubin and (EZ)-bilirubin as a result of photochemical isomerization after irradiation of both (EZ)-bilirubin and (ZE)-bilirubin bound to human serum albumin

For details see the text.

short time, i.e. 6, 12, 24 or 36 s, the peak of (ZZ)-bilirubin decreased exponentially, with a half-life of 23.8 s, and at the same time a peak of (ZE)-bilirubin appeared. A summary of the photochemical reactions plotted against time on a linear scale is shown in Fig. 1(a). A good quantitative correlation between the sum of the disappearance of (ZZ)-bilirubin and the appearance of (ZE)-bilirubin was observed within the period of the experiment (Table 1). During the period of the experiment, the appearance of peaks of (EZ)-bilirubin and (EZ)-cyclobilirubin was negligible.

Transformation of (ZZ)-bilirubin into (EZ)-cyclobilirubin (reaction ii)

Kinetics for reaction (ii) are as follows.

$$\begin{aligned}
 \frac{dB}{dt} &= k_2 D - (k_1 + K) B \\
 \frac{dD}{dt} &= k_1 B - k_2 D
 \end{aligned}$$

For Laplace conversion:

$$\begin{aligned}
 (s + k_1 + K) Q_2 - k_2 Q_2 &= B_0 \\
 k_1 Q_1 - (s + k_2) Q_2 &= 0
 \end{aligned}$$

$$\begin{aligned}
 Q_1 &= \frac{\begin{vmatrix} B_0 & -k_2 \\ 0 & -(s + k_2) \end{vmatrix}}{\begin{vmatrix} s + k_1 + K & -k_2 \\ k_1 & -(s + k_2) \end{vmatrix}} \\
 &= \frac{-B_0(s + k_2)}{-(s + k_1 + K)(s + k_2) + k_1 k_2} \\
 &= \frac{B_0 s + k_2 B_0}{s^2 + (k_1 + k_2 + K)s + k_2 K} \quad (5)
 \end{aligned}$$

$$\begin{aligned}
 Q_2 &= \frac{\begin{vmatrix} s + k_1 + K & B_0 \\ k_1 & 0 \end{vmatrix}}{\begin{vmatrix} s + k_1 + K & -k_2 \\ k_1 & -(s + k_2) \end{vmatrix}} \\
 &= \frac{k_1 B_0}{s^2 + (k_1 + k_2 + K)s + k_2 K} \quad (6)
 \end{aligned}$$

If $B = A_1 e^{-\alpha t} + A_2 e^{-\beta t}$ and Laplace conversion takes place:

$$Q_1 = \frac{A_1}{s + \alpha} + \frac{A_2}{s + \beta} = \frac{(A_1 + A_2)s + A_1\beta + A_2\alpha}{s^2 + (\alpha + \beta)s + \alpha\beta} \quad (7)$$

From eqns. (5) and (7):

$$A_1 + A_2 = B_0 \quad (8)$$

$$A_1\beta + A_2\alpha = k_2 B_0 \quad (9)$$

Table 1. *Initial photochemical isomerization of (ZZ)-bilirubin*
For details see the text.

Duration of irradiation (s)	(ZZ)-Bilirubin (B) (%)	(ZE)-Bilirubin (D) (%)
0	100	0
6	87	13
12	80	20
24	76	24
36	77	23

Table 2. *Further photochemical isomerization of (ZZ)-bilirubin*
For details see the text.

Duration of irradiation (s)	(ZE)-Cyclobilirubin (C) (%)	(ZZ)-Bilirubin (B) (%)	(ZE)-Bilirubin (D) (%)
0	0	100	0
60	1	72	27
120	4	70	26
180	5	69	26
240	10	65	25

$$\alpha + \beta = k_1 + k_2 + K \quad (10)$$

$$\alpha\beta = k_2K \quad (11)$$

$$(\alpha - \beta)A_2 = (\beta - k_2)B_0$$

$$A_2 = \frac{(\beta - k_2)B_0}{\alpha - \beta}$$

$$A_1 = \frac{(\alpha - k_2)B_0}{\alpha - \beta}$$

From eqns. (10) and (11):

$$(k_1 + k_2 + K - \beta)\beta = k_2K$$

$$(k_1 + k_2)\beta + \beta K = k_2K$$

$$K = \frac{(k_1 + k_2)\beta}{k_2 - \beta}$$

$$= \frac{1.39 \times 10^{-1} \times 5.92 \times 10^{-4}}{1.06 \times 10^{-1} - 5.92 \times 10^{-4}}$$

$$= \frac{8.23 \times 10^{-5}}{1.06 \times 10^{-1}} = 7.76 \times 10^{-4}$$

Therefore:

$$B = \frac{(\alpha - k_2)B_0}{\alpha - \beta} e^{-\alpha t} + \frac{(\beta - k_2)B_0}{\alpha - \beta} e^{-\beta t}$$

When $\alpha \gg \beta$, and t is sufficiently long (Fig 3):

$$B \doteq \frac{(\beta - k_2)B_0}{\alpha - \beta} e^{-\beta t}$$

$$\beta = \frac{\ln 2}{1170} = 5.92 \times 10^{-4}$$

$$\alpha + \beta = k_1 + k_2 + K \quad (10)$$

$$\alpha\beta = k_2K \quad (11)$$

From reaction (i):

$$k_1 + k_2 = 1.39 \times 10^{-1}$$

$$k_2 = 1.06 \times 10^{-1}$$

A further photochemical experiment was also carried out as described above except that the duration of photoradiation was for 60, 120, 180 or 240s. In addition to the decrease in (ZZ)-bilirubin and the appearance of (ZE)-bilirubin, a peak of (EZ)-cyclobilirubin appeared and increased. A good quantitative correlation between the disappearance of (ZZ)-bilirubin and the appearance of (ZE)-bilirubin and (EZ)-cyclobilirubin was also observed within the period of the experiment (Table 2). The appearance of these peaks of (EZ)-bilirubin and (EE)-cyclobilirubin was negligible.

Kinetic study of photochemical experiments of (EZ)- and (ZE)-bilirubin-enriched human serum albumin in solution (reaction iii)

Kinetics for reaction (iii) are as follows.

$$\frac{dA}{dt} = -(k_3 + k_5)A$$

$$\frac{dC}{dt} = k_5 A$$

$$A = A_0 e^{-(k_3 + k_5)t}$$

$$C = -\frac{k_5 A_0}{k_3 + k_5} e^{-(k_3 + k_5)t} + \frac{k_5 A_0}{k_3 + k_5} + C_0$$

where A and C are percentages of A and C respectively.

$$k_3 + k_5 = \frac{\ln 2}{12} = 5.78 \times 10^{-2}$$

When $t \rightarrow \infty$:

$$C \rightarrow \frac{k_5 A_0}{k_3 + k_5} + C_0$$

$$C_0 = 4, \quad A_0 = 23$$

$$\frac{k_5 A_0}{k_3 + k_5} + C_0 = 15$$

$$\frac{k_5 A_0}{k_3 + k_5} = 11$$

$$\frac{k_5}{k_3 + k_5} = \frac{11}{23}$$

$$k_5 = 2.76 \times 10^{-2}$$

$$k_3 = 3.02 \times 10^{-2}$$

$$K \ll k_5$$

Therefore:

$$K \doteq k_4$$

A summary of the photochemical changes plotted against time on a linear scale is shown in Fig. 1(b).

Within 36s of irradiation, the peak of (EZ)-bilirubin decreased single-exponentially. The slopes of the disappearance curves of these two peaks were parallel, and at the same time (EZ)-cyclobilirubin and (ZZ)-bilirubin, derived from (EZ)-bilirubin and (EZ)-/(ZE)-bilirubin respectively, increased in parallel within 24s (Fig. 4).

Fig. 2. Change of $1 - D/24$ plotted against time on a semi-logarithmic scale

Fig. 3. Changes in percentages of (ZZ)-bilirubin and (EZ)-cyclobilirubin and (ZE)-bilirubin as a result of photochemical isomerization after irradiation of (ZZ)-bilirubin. For details see the text.

Table 3. Photochemical isomerization of (EZ)- and (ZZ)-bilirubin
For details see the text.

Duration of irradiation (s)	(EZ)-Cyclobilirubin (C) (%)	(EZ)-Bilirubin (A) (%)	(ZZ)-Bilirubin (B) (%)	(ZE)-Bilirubin (D) (%)
0	4	23	13	60
6	9	16	36	39
24	15	6	53	26
36	15	3	57	25

Fig. 4. Changes in percentages of (EZ)-bilirubin, (ZE)-bilirubin, (EZ)-cyclobilirubin and (ZZ)-bilirubin as a result of photochemical isomerization after irradiation of both (EZ)-bilirubin and (ZE)-bilirubin bound to human serum albumin

For details see the text.

The half-lives of the (EZ)- and (ZE)-bilirubins, i.e. the rates of photochemical transformations into (EZ)-cyclobilirubin and (ZZ)-bilirubin, were 12 and 13.5s respectively. A good quantitative correlation between the disappearance of (EZ)- and (ZE)-bilirubin and the appearance of (EZ)-cyclobilirubin and (ZZ)-bilirubin was observed within the period of experiment (Table 3).

A summary of photochemical transformations from (EZ)-bilirubin into (EZ)-cyclobilirubin or (ZZ)-bilirubin plotted against time on a semi-logarithmic scale is shown in Fig. 4.

The principal pathways for the formation of bilirubin photoproducts when bound to human serum albumin therefore can be summarized as follows:

where k_1, k_2, k_3, k_4 and k_5 are the relative rate constants for the individual photochemical reactions. A summary of the values obtained from the kinetic study of photochemical experiments described above is shown in Table 4.

In conclusion, in photochemical experiments *in vitro* with (EZ)-bilirubin bound to human serum albumin in solution, the sum of the relative rate constants of photochemical transformations of (EZ)-bilirubin into both (EZ)-cyclobilirubin and (ZZ)-bilirubin (with a significant preference for the former) is proved to be considerably larger, by two orders, than that of the transformations of

Table 4 Summary of the relative rate constants for the individual photochemical reaction

$$\begin{aligned}
 k_1 &= 3.34 \times 10^{-2} \text{ s}^{-1} \\
 k_2 &= 1.06 \times 10^{-1} \text{ s}^{-1} \\
 k_3 &= 3.02 \times 10^{-2} \text{ s}^{-1} \\
 k_4 &= 7.76 \times 10^{-4} \text{ s}^{-1} \\
 k_5 &= 2.76 \times 10^{-2} \text{ s}^{-1} \\
 K &\equiv k_4 (\because K \ll k_5)
 \end{aligned}$$

(ZZ)-bilirubin into (EZ)-bilirubin. Therefore only one of the geometrical isomers, namely (ZE)-bilirubin, is apparently formed. It was concluded that (EZ)-bilirubin photochemically undergoes (EZ)-cyclization while bound to its high-affinity site on human serum albumin, and is indubitably an intermediate in the transformation of (ZZ)-bilirubin into (EZ)-cyclobilirubin.

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